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Quality assessment and occurrence of resistant Bacterial pathogens in Gelatin production in Leather and Pharmaceutical industries and their effect to humans

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ABSTRACT

The present study was undertaken to determine the bacteriological analysis of unsanitized quality gelatin production in leather processing in comparison pharmaceutical gelatin from different sources within the leather and pharmaceutical industries in Zaria and Kaduna environ respectively. A total of sixteen samples were designated with label on both leather and pharmaceutical gelatin (S_1-S_8). Bacteriological quality of the leather and pharmaceutical gelatin were also analyzed in-situ. The following organisms were

identified viz; *Bacillus* sp, *Klebsiella* sp, *Prosteus* sp, *Enterobacter* sp, *Pseudomonas* sp and *Clostridium* sp. The percentage resistant occurrences of the pathogens in respective gelatin water samples were also noted. Only few of the samples comply with the WHO standard while other could not. There were higher contaminants in leather processing than for pharmaceutical gelatin processing. The most frequent resistants sp were the spore formers ie *Bacillus* and *Clostridium* sp. *Bacillus* sp ranges from (31.57-39.53%) followed by *Clostrdium* sp (23.68-27.91%) and the least were found(9.30-13.15%) from *Enterobacter* and *Pseudomonas* sp. The presence of these pathogens has indeed become hindrance and problematic in the production of good standard and technical grade gelatin from leather and pharmaceutical industries. Since gelatin served as a bi- product of tissue skin from hides and bone of cow is commonly use during the production of various food processing industries. It is a must to incorporate in a well founded management system by its highly sanitized condition and hence are generally accepted among customers, their usefulness for quality control management is often limited both economically and scientifically. The case study was concentrated mainly on the frequency and occurrence of bacterial pathogens and other less resistant member of pathogens which are prompt to potent causative agent in food spoilage, poisoning, food borne disease and their health hazard to humans are discussed in-situ.

Keywords: Leather gelatin, pharmaceutical gelatin, bacterial pathogens

1. INTRODUCTION

The bacteriological quality of gelatin is of great importance, as it is applied for its gelling and stabilizing properties in the food industry (confectionery products, dairy products, etc.) and the pharmaceutical industry (hard and soft capsules, tablets, etc.) and in the production of photographic films, matches, glues, etc. Gelatin is a proteinaceous colloid and is extracted from animal connective tissue during a multistage process which involves. Besides the actual extraction, a chemical treatment, purification, and drying of the extracts of skin and bones mainly of bovine or porcine origin are used for this purpose. These raw materials are collected from slaughterhouses, butcher shops, or other plants dealing with animal raw materials.

Gelatin is the product of denuturation or disintegration of collagen. It is extracted from degreased and demineralised crushed skin tissue/bone (termed as Ossein). Following their acid and alkali treatment, in the form of weak liquor (cole and Rebertis, 1997). The weak liquors is concentrated and dried to yield gelatin in the form of granules because of its unique properties such as solubility, solution, viscosity and thermally reversible gelatin properties. Gelatin find used in pharmaceutical, food, photographic and other industries such as cosmetic and safety matches.

Bacterial contamination during gelatin production is of great concern. Gelatin is a proteinaceous colloid that is extracted from animal connective tissue as, for example procine or bovine skin and bones. In a previous study, contamination of a gelatin production process, with aerobic endospore formers was demonstrated by De cleark and Devos, (2002), contamination of industrial plants and products with aerobic endospore formers is a frequently reported problem (Lingarthurai and Vellathurai, 2010). Their ubiquitous occurrence in combination with their wide nutritional versatility, wide pH and temperature ranges for growth and the formation of endospores that are much resistant to heat, chemical, irradiation and desiccation than vegetative forms (Set low, 1998) make this group of bacterial an ever-present problem in different industries. Extreme temperature and pH conditions during gelatin manufacturing, with ultra high temperature (UHT) treatment and drying of gelatin should minimized microbial contamination at the end product, but thermo tolerant, aerobic endospore-forming bacterial, attribute to the genus *Bacillus* and related genera, were shown of persistent in the semi-final product.

Bacterial contamination of gelatin may affect its safety and/ or quality in use. Indeed, some of these contamination posses pathogenic properties of man and thus are a threat to human health in food and pharmaceutical applications. Furthermore, gelatin contaminants have been shown to exhibit gelatin activity (De clerk and Devos, 2002). And therefore negatively affect the viscosity and gelling capacity of the product. As a result of this gelatin liquefaction, nutrients may become available or gelatin negative contaminant promoting her growth. Detection, in the production line, of endsopore-forming contaminants that are known to have the potential to survive the production process, would be of great value for quality control management.

Contamination of a gelatin production process with a variety of gram-positive and gram-negative bacteria was reported by De clerck and Devos, (2002). However, extreme temperature and pH conditions during the manufacturing, ultrahigh-temperature (UHT) treatment, and drying of the gelatin extracts should guarantee the microbial sterility of the end product. Nevertheless, quality controls testing at gelatin-producing factories have indicated that thermotolerant, aerobic, endospore-forming bacteria may persist in the final product (Paul Stevens, personal communication).

In general contamination of industrial plants and products with aerobe endosporeformers is a widespread problem. The ubiquitous occurrence of these bacteria in combination with their wide nutritional versatility and wide pH and temperature ranges for the growth and formation of endospores, which are much more resistant to heat, chemicals, irradiation, and desiccation than vegetative forms (Setlow, 1994), makes this group of bacteria an ever-present problem in different industries (Brown, 2000) and (Heyndrick and Scheldeman, 2002). *Bacillus licheniformis*, members of the *Bacillus cereus* group, *Bacillus coagulans*, *Bacillus fumarioli*, *Bacillus badius*, *Bacillus subtilis*, *Brevibacillus agri*, *Alicyclobacillus acidocaldarius*, and *Paenibacillus cookii* were found to be contaminants in a Belgian gelatin production process (Declerck and Devos, 2002).

Gelating being an excellent nutrient for most bacterial, the manufacturing process has to be extremely carefully voided in term of decontamination by pathogens, since most of the gelatin manufacture is used in food industry.

In this present work, it was concentrated mainly on the occurrence of bacterial pathogens and other member of pathogens that seem to be existing and proving to be resistant at all stages of gelatin production, which are considered as potent causative agent to food spoilage and food borne disease.

2. MATERIALS AND METHODS

2.1. Sampling and sample preparation

A gelatin production line using porcine skin as raw materials, from Nigerian institute leather and science technology (Nilest) samara Zaria/gelatin pharmaceutical industry to produce the highest quality of gelatin for application in pharmaceutical products. Sampled were taken at various stages of the production chain. The samples used in this present investigation were collected from the water used, in the manufacturing process, skin/ossein before and after pre-treatment, weak liquor, strong liquor (concentrated weak liquor wet noodle at different zones of drying and finished products).

2.2. Isolation of bacterial from various stages of production

Standard procedures were used for the analysis of the bacterial pathogens as ascribe by Fawole and Oso, (2004) within 48 hours of sampling. isolation of bacteria commenced in-situ. Serial dilution method was adopted for both gelatin and liquid samples (i.e. water and liquor) were enriched using nutrient broth (NB) as dilent. Each of the dilution (0.1ml) was spread on nutrient agar (NA) plates in duplicate, which were incubated for 48 hours at 28^{o}C. Distinct colonies were picked and re- inoculated into agar slant to obtain pure isolates. It was then counted by their percentage differences using calculative method as ascribed by (Aneja, 2007).

2.3. Subcuturing and maintenance of bacterial isolates

For short term bacterial culture storage, bacteria were subculture on (NA) slant and maintained at 4^{o}C where as for long term storage mid-exponential phase isolates were taken in nutrient broth and covered with 100% glycerol and kept at- 20^{o}C (Fawole and Oso, 2004).

2.4. Identification and biochemical characterization of bacterial strains

Isolated colonies after purification were initially gram stained. By using bergey's manual of determination of bacteriology (9th edition), the isolates were biochemically characterized and identified as follows. Catalax test oxidax test, spore staining, starch hydrolysis, citrate utilization test, methyl red vogas prokauer test (MR-VP), nitrate reduction test gelatin liquefaction test, triple sugar iron test, lactose/glucose fermentation, indole production test, urease test , motility test etc (Bryan 1993).

3. RESULTS AND DISCUSSION

Six different bacteria were isolated viz; *Bacillus* sp, *Klebsiella* sp, *Proteus* sp, *Entero-bacter*, *Pseudomonas* sp and *Clostridium* sp., as contaminant from leather and pharmaceutical industries. These bacteria were screened and characterized owing to the nature of their survival, mode of gelatin source assimilation and their morphology as seen in table 1: In table 2 and 3 the present report gave a survey of bacterial general species occurrence frequently in gelatin production. Particular attention was focused mostly on those strains identified namely: *Bacillus* sp, *Klebsiella* sp, *Proteus* sp, *Entero-bacter*, *Pseudomonas* sp and *Clostridium* sp from different stages of gelatin production. To this effect, the observed water use during the process contained little or no bacterial (bacterial free). The possible explanation could be that there was proper water treatment for gelatin production ascribed by WHO guide line for the portability of water (WHO, 1973). Therefore, the possibility of occurrence of these pathogens through the water used during gelatin production was found to be free of bacterial pathogens. The result in table 2 and 3 also show the percentage differences of frequent bacterial sp in treatment of ossein/skin tissue sample falls within the range of (31.57-39.535%) *Bacillus* sp being the highest, followed by *Clostridium* sp (23-27.91%), and least was found within the range of (9.30-13.15%) *Enterobacter* sp. *Proteus* sp was dully

observed but due to its swampy nature in counting under the condition of the study was impossible. *Pseudomonas* sp and *Kleibsiella* sp were also noted being fair in gelatin assimilation as shown in table 1: However, it was also found that ossein/tissue skin sample after treatment did not show the presence of these pathogens. This may be on the condition due to drastic change during the pretreatment at low pH was maintained and increased considerable after the pre-treatment. It was also observed in weak liquor, *Bacillus* sp and *Clostridium* sp have the highest count ranging from (4-6) and (3-4) followed by *Klebsiella* sp (2-3) and the least was found by *Pseudomonas* sp and *Enterobacter* sp of gelatin production. This could be attributed to the addition of appropriate preservatives in weak liquor which might have inhibited the growth of *Enteriobacter* sp or otherwise. While the other two treatments (strong liquor and drying) zone none of these pathogens were observed due to proper sterilization prior to the concentration of weak liquor to strong liquor.

The microbial analysis of the plant using swabs from leather and pharmaceutical gelatin production showing the occurrence frequency count range of (2-3) *Bacillus* sp, *Clostridium* sp (1-2), *Pseudomonas* sp (1-1), *Enterobacter* sp (1-1), *Klebsiella* sp (1-1) except *Proteus* sp.

Table 1

Morphology and biochemical characterization of the bacteria isolates

Character	***Bacillius* sp**	***Pseudomonas* sp**	***Proteus mirabilis***	***Klebsiella* sp**	***Enterobacter* sp**	***Clostridium* sp**
Umbonate colony	-	+	-	-	-	-
Trimethlamine odour	-	+	-	-	-	-
Rod/Cocci	Rods	Rods	Rods	Rods	Rods	Drump shave rods
Pigment/production	-	+	-	-	-	-
Motility	+	+	+	-	-	+
Swarming	-	-	+	+	-	-
Sporeformation	+	-	-	-	-	+
Gram reaction	+	-	-	-	-	+
Aerobid (A) facuta	A/FA	A	F/A	F/A	F.A	F.A
Catalose activities	+	+	+	+	+	+
Oxidase activity	+	+	+	+	+	+
Gelatin liquid faction	+	+/-	+	+/-	+/-	+
Indole production	ND	ND	+/-	+/-	-	-
Citrateutilization	-	-	+/-	+/-	+	+
Urease activities	ND	ND	+	+	+/-	+/-
H2s. production	ND	ND	+	-	-	+
Methyl red	ND	ND	+	-	+	+
Vogesproskau	ND	ND	+/-	+/-	-	-
Lactose	+	-	-	+	+	+
Glucose	+	+/-	+	+	+/-	+

Key=characters for which different general score positive (+) negative (-) ND = Not done

Among the finished product pure grade edible grade leather and pharmaceutical grade gelatin, all were free of any pathogens. In the case of technical grade gelatin, the occurrence frequency of *Bacillius* sp, *Clostridium* sp , *Pseudomonas* sp, *Klebsiella* sp, and *Enterobacter* sp were ranged between (31.15- 39.53%), (23.68-27.9%), (11.62-15.78%), (9.30-15.78%) and (9.30-13.15%) respectively. The isolated and identified organisms in every water treatment for gelatin production has shown a considerable occurrence in weak liquor and none was recovered in strong liquor or the drying zone due to sterilization. In spite of the high resistant spores exhibited

by the species when tested on thermal death point pretreatment showed their survival was limited. Hence, their presence in ossein/ tissue skin treatment has no economical value to industries of concerned as well as human or otherwise.

Since these pathogens are generally present in this current investigation in in both leather and pharmaceutical production of gelatin and their presence in gelatin liquefaction tested positive as shown in table 1. It might not be surprising in spite of all takes to ensure the purity of gelatin in leather and pharmaceutical industries regarding to consumption prove abortive. Their presence in leather and pharmaceutical production could be regarded as an adulterated case due to improper sanitary check. These impromptu pathogens might found themselves in localized fashion in the pre- gelatin production stage before sale to consumers. Once they get to the consumer by ingestion into the system, it turns to be hazardous to humans due to their presence.

In this study and it should be worthwhile to know their pathogen city (disease forming potency) the occurrence and incubation period if necessary.

Bacillus sp includes large aerobic, gram-positive rods occurring in chains. Most members of this genus are saprophytic organisms prevalent in water, air or the worker's hand may be transmitted in to liquefied gelatin. *Bacillus cereus* and *Bacillus subtilis* are insect pathogens. *B cereus* can grow in foods and produce an enterotoxin or an emetic toxin and cause food poisoning. Such organisms may occasionally produce disease in immunocompromised humans (eg, meningitis, endocarditis, endophthalmitis, conjunctivitis, or acute gastroenteritis). *B anthracis,* which causes anthrax, is the principal pathogen of the genus (Lingarthurai and Vellathurai, 2010).

Table 2

Occurrence frequency of pathogen at different stage of gelatin production in Pharmaceutical industries

Bateria Isolates	S1	S2	S3	S4	S5	S6	S7	S8	Total	(%)
Pseudomonas sp	_	4	_	1	_	_	1	_	6	15.78
Enterobacter sp	_	2	_	2	_	_	1	_	5	13.15
Proteus sp	_		_	_	_	_		_		_
Bacillus sp	_	6	_	4	_	_	2	_	12	31.57
Clostridium sp	_	5	_	3	_	_	1	_	9	23.68
Klebsiella sp	_	3	_	2	_	_	1	_	6	15.78

Total 38

Key

S1 = water used during the process
S2 = Skin tissue before pretreatment
S3 = Skin liquor
S4. = weak liquor
S5 = strong liquor
S6 = drying zone
S7. = swabs of plant
S8 = finished products (pure industrial and edible pharmaceutical grade gelatin)

Clostridium sp are usually wider than the diameter of the rods in which they are formed. In the various species, the spore is placed centrally, sub-terminally, or terminally. Most species of *clostridia* are motile and possess peritrichous flagella. *Clostridia* are anaerobes and grow under anaerobic conditions; a few species are aerotolerant and will also grow in ambient air. In general, the clostridia grow well on the blood-enriched media as well as gelatin media. They are used to grow anaerobes and on other media used to culture anaerobes as well (Aroujo, 2001).

Clostridium botulinum, which causes botulism, is worldwide in distribution; it is found in soil, air and might also be found from the workers in gelatin industry. *C botulinum*s are distinguished by the antigenic type of toxin they produce. Spores of the organism are highly resistant to heat, withstanding 100°C for several hours. Heat resistance is diminished at acid pH or high salt concentration. Although *C botulinum* types A and B have been implicated in cases of wound infection and botulism, most often the illness is not an

infection. Rather, it is an intoxication resulting from the ingestion of food in which *C botulinum* has grown and produced toxin. The most common offenders are bottle cap wet gelatin, vacuum-packed, or canned alkaline foods that are eaten without cooking. In such foods, spores of *C botulinum* germinate; under anaerobic conditions, vegetative forms grow and produce toxin (Aroujo, 2001).

The toxin acts by blocking release of acetylcholine at synapses and neuromuscular junctions. The electromyogram and edrophonium strength tests are typical. *C tetani* is not an invasive organism. The infection remains strictly localized in the area of devitalized tissue (wound, burn, injury, umbilical stump, surgical suture) into which the spores have been introduced. The volume of infected tissue is small, and the disease is almost entirely a toxemia.

Pseudomonas sp is Gram negative, rod shaped opportunistic pathogen, which could be found in food industries (diary, gelatin etc), soil and water. According to United States National Nosocomial Infection Surveillance System, it accounts for 16.1% of all nosocomial infections and ranked second among Gram negative pathogens (Khan et al., 2008).

Healthy adults rarely encounter its infection but main target is people having compromised immune system including HIV infections. The infection ranges from self-limiting folliculitis to life threatening bacteremia, wound related morbidity, septicemia, skin infections, otitis media, ecthymegangrenosum, the black necrotic lesion, endocarditis, corneal ulceration and device related infections (Ranjan et al., 2010). It is the third leading cause of 12 % of hospital-acquired urinary tract infections, upper and lower respiratory tract infections like cystic fibrosis that is associated with high mortality rate in immune-compromised patients (Todar, 2011). Gender-wise prevalence showed 61.78% male and 38.22% females were infected by P. aeruginosa (Todar, 2011). Souli and Colleagues (2008) published data from 23 countries on the European Antimicrobial Resistance Surveillance System and it was shown that 18% of all isolates were multidrug resistant P. aeruginosa strains. Ullah and Colleagues (2012) carried out a study in Islamabad and showed that P. aeruginosa is 94% resistant to Chloramphenicol, 88% to Colistin /sulphate, 73% to Tetracycline and 3% to Imipenem. The resistance against the newly tested drugs is still evolving as P. aeruginosa is highly resistant to antibiotics, both at the genetic level and as a result of living in multilayered and complex biofilm (Strateva and Yourdonov, 2009).

Table 3

Occurrence frequency of pathogen at different stage of Gelatin production in leather processing industries

Bacterial Isolates	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Total**	**(%)**
Pseudomonas sp	_	3	_	1	_	_	1	_	5	11.62
Enterobacter sp	_	2	_	1	_	_	1	_	4	9.30
Proteus sp	-	swampy	-	swampy	_	_	swampy	_		_
Bacillus sp	_	8	_	6	_	_	3	_	17	39.53
Clostridium sp	_	6	_	4	_	_	2	_	12	27.91
Klebsiella sp	_	4	_	3	_	_	1	_	7	16.28

Total 43

Key

S1 = water used during the process
S2 = Skin tissue before pretreatment
S3 = Skin liquor
S4. = weak liquor
S5 = strong liquor
S6 = drying zone
S7. = swabs of plant
S8 = finished products (pure edible, industrial leather grade gelatin)

Enterobacter sp is a motile, Gram-negative, non-spore-forming, and rod-shaped bacterium that has been found in infant formulas as a contaminant. *Enterobacter* species are biochemically similar to *Klebsiella*; unlike *Klebsiella*, however, *Enterobacter* is ornithine positive. *Enterobacter sakazakii* has been found to be more resistant to osmotic and dry stress than other members of Enterobacteriaceae family.The reservoir for *E. sakazakii* is unknown. Various environmental samples (surface water, soil, mud, bird faeces) have tested negative. It has been identified in the guts of certain flies. The organism has been frequently identified in factories that produce milk powder, gelatin powder and other food substances in households and commercialized products.

Enterobacter sp has been associated with sporadic cases or small outbreaks of sepsis, meningitis, cerebritis and necrotizing enterocolitis. Most of the infections are seen in low-birth-weight infants (i.e., less than 2 kg) or infants born prematurely (i.e., less than 37 weeks of gestation).Mortality has been reported to be as high as 50% but has decreased to less than 20% in recent years (WHO/FAO, 2004).

Klebsiella sp and *Proteus* sp are non-spore formers; their possibility of occurrence in the finished products (gelatin) is very low. Also these are facultative aerobe hence their presence in air cannot be rule out. However, maintenance of sterile working environment is suggested as a preventive measure. They may play a more significant role in contamination, spoilage and ill health as well. *Klebsiella* sp has also been identified as pathogen and often indicated in pneumonia, notably of the form characterized by multiple cavitations of lungs. They can also cause meningitis, Otitis and sinusitis (Cruck Shamic et al., 1980). They are present in sputum, thus are air borne and can be spread by contact hence transmission through hand used of utensils. The organism is destroyed at 60ºC in 20 minutes and by common disinfectant. Incubation is also at 37ºC at a pH range of 6.8 to 7.2 (Mohammed et al., 2009). These species were isolated and identified as gelatin positive. Also the occurrence of *Klebsiella* sp and *Proteus* sp was much lower than that of *Clostridium* sp and *Bacillus* sp. Thus special preventive measure is suggested against contamination of product by the non-spores former and sterile working conditions and instruments are suggested to prevent their occurrence. Also since they are facultative aerobic, their population in the initial stage can be controlled by proper aerobic of ossein/tissue skin during the maturation period.

The finished products barring technical grade gelatin were free of any bacterial. Thus, in accordance with the international commission on microbiological specification for foods (IC MSD, 1988). The occurrence of these pathogens in technical grade gelatin is in significant as it is never used for consumption.

4. CONCLUSION

The pathogens isolated and identified from different samples during present investigation were *Bacillus* sp, *Pseudomonas* sp, *Proteus* sp, *Klebsiella* sp, Entero-bacter sp and *Clostridium* sp. Since the water used during the process were free of these pathogens. Their occurrence due to water can be rule out under the present study. The occurrence of *Clostridium* sp and *Bacillius* sp were identified to have higher percentage. While other like *Klebsiella* sp, *Pseudomonas* sp etc in weak liquor was very significant. Since this denoted some cross contamination as they were about in the previous step i.e. ossein/tissue skin after treatment, but their growth was again checked in the next stage i.e. in strong liquor where all sorts of bacterial were absent. The drying conditions of the plant did not favour the growth of *Klebsiella* sp, *Proteus* sp and *Pseudomonas* sp, but *Clostridium* sp and *Bacillus* sp were present. Barring technical grade gelatin, the finished products had excellent microbial quality. Since they were free of both aerobic and anaerobic bacterial technical grade gelatin was produced during plant breakdown situation, power failure or when the plant malfunctioned due to some reason and hence the high frequency of *Clostridium* sp and *Bacillus* sp in the sample. Also microbial quality of technical grade gelatin is significantly important from the public health point of view, since they are used for human or animal consumption.

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